ORIGINAL ARTICLE VIROLOGY

Outbreak of hand, foot and mouth disease/herpangina associated with coxsackievirus A6 and A10 infections in 2010, France: a large citywide, prospective observational study

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Abstract

Hand, foot and mouth disease (HFMD) and herpangina (HA) are frequently caused by several distinct serotypes belonging to the human enterovirus A species (HEVA). Enterovirus 71 is considered as a significant public health threat because of rare but fatal neurological complications. A sentinel surveillance system involving paediatricians from Clermont-Ferrand (France) was set up to determine the clinical and epidemiological characteristics of HFMD/HA associated with enterovirus infections. A standardized report form was used to collect demographic and clinical data. Throat or buccal specimens were obtained prospectively and tested for the presence of enteroviruses. The frequency of HEVA serotypes was determined by genotyping. Phylogenetic relationships were analysed to identify potential new virus variants. From 1 April to 31 December 2010, a total of 222 children were enrolled. The predominant clinical presentation was HA (63.8%) and this was frequently associated with clinical signs of HFMD (48%). An enterovirus infection was diagnosed in 143 (64.4%) patients and serotype identification was achieved in 141/143 (98.6%). The predominant serotypes were coxsackievirus A10 (39.9%) and A6 (28%), followed by coxsackievirus A16 (17.5%) and enterovirus 71 (6.3%). Fever was observed in 115 (80.4%) children. No patient had neurological complications. Coxsackievirus A10 and A6 strains involved in the outbreak were consistently genetically related with those detected earlier in Finland and constituted distinct European lineages. Although several enterovirus serotypes have been involved in HFMD/HA cases, the outbreak described in this population survey was caused by coxsackievirus A6 and coxsackievirus A10, the third dual outbreak in Europe in the last 3 years.

Keywords: Enterovirus 71, enterovirus genotyping, hand, foot and mouth disease, human enterovirus A, molecular epidemiology
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Introduction

Hand, foot and mouth disease (HFMD) is a common childhood disorder that typically presents as a brief, febrile illness characterized by the association of oral ulcerations (enanthema) and vesicular rash (exanthema) on the palms, soles and/or buttocks. In herpangina (HA), the enanthema resembles that in HFMD except that the ulcerative lesions are predominantly located on the posterior oropharyngeal structures [1].

Human enteroviruses (HEVs) are the aetiological agents involved in most cases of both HFMD and HA, in particular the serotypes belonging to the A species (HEVA), a group of 22 genetically related serotypes [2]. Two serotypes, coxsackievirus A16 (CV-A16) and enterovirus 71 (EV-71), cause most of the epidemics worldwide [3–5]. Epidemiological studies of HFMD/HA outbreaks in Europe, Southeast Asia and North America showed that CV-A16 and a number of other HEVA serotypes usually cause self-limiting infections [6–10]. In contrast, during outbreaks involving EV-71, neurological

complications (acute flaccid paralysis, brainstem encephalitis associated with cardiopulmonary oedema) may occur. In a prospective study of HFMD epidemics in Sarawak, Malaysia over 7 years, 10–30% of children with HFMD manifestations caused by EV-71 developed central nervous system complications of which brainstem encephalitis (58%) was the most frequent presentation, for review see [11]. Since 1997, epidemic waves of EV-71 infections have swept countries in the Asia Pacific region [6]. Circulating EV-71 strains are clustered in genogroups B and C, and in sub-genogroups B0–B5 and C1–C5 [12–14], and changes in the predominant sub-genogroup are observed at each epidemic wave [6].

In Europe, outbreaks of EV-71 neurological infections with high case fatality rates were recorded in the 1970s [15,16]. In recent years, EV-71 genogroups C1 and C2 virus strains have been detected in several countries and an increased incidence of infections was observed in 2007 [14,17,18]. Infections resulting in fatal outcome have also been reported [19]. There is a need for reinforced surveillance of HFMD/HA in children and rapid diagnosis to differentiate benign HEVA serotypes from EV-71.

We set up a citywide sentinel surveillance system of HFMD/HA, involving a university paediatric emergency unit and paediatricians in private practice. We performed a prospective observational study to assess the relative frequency of HEVA infections, to identify the different associated serotypes, and to establish well-defined procedures for future survey studies at a national level.

Patients and Methods

Patients and data collection

From 1 April to 31 December 2010, sentinel paediatricians were requested to collect clinical specimens from patients presenting with HFMD, HA or gingivostomatitis. Informed consent was obtained from each child's accompanying parent.

Throat or buccal swabs (when oral ulcerations were present) were collected using a universal viral transport system (Becton Dickinson, Sparks, MD, USA). Each specimen along with a standardized report form was prospectively sent to the virology laboratory for detection of the EV genome, genotyping and viral culture. The form recorded information on the patient's demographics and clinical findings. Ten clinical manifestations (fever, vesicular rash of palms, soles or other localization, oral ulcers, gingivostomatitis, HA, digestive/respiratory/ear nose and throat/neurological signs) were recorded. Fever was a rectal temperature higher than 38°C. HFMD was defined as oral ulcers on the tongue and buccal mucosa with vesicular rash on the hands, feet, knees or buttocks. HA was defined as oral ulceration affecting predominantly the anterior tonsillar pillars, soft palate, or the uvula. Gingivostomatitis was defined as oral ulcers on the buccal and gingival mucosa. Neurological manifestations were defined as clinical signs of meningitis, encephalitis or convulsions. Environmental data (contact with persons presenting similar symptoms, child-care arrangements) were also recorded on the form. Results of viral testing were sent prospectively to the physicians with an extra copy for the patient. Sentinel paediatricians were informed monthly of the results of the prospective surveillance.

Since 2000, hospitalized cases of EV infections in France have been voluntarily reported through a laboratory network to the national Institute for Public Health. We compared the epidemiological data from the local sentinel surveillance system with those collected at a national level.

Enterovirus detection and genotyping

Viral RNA was extracted from 200 μL of the universal viral transport medium on the NucliSens® EasyMAG™ automated system (bioMérieux, Marcy l'Etoile, France). The EV genome was detected by real-time NASBA detection using the NucliSens EasyQ® Enterovirus kit (bioMérieux). Using the same RNA extracts, genotyping of EV strains was performed with a procedure similar to that described previously for the identification of HEVB serotypes [20]. After cDNA synthesis, the complete 1D gene sequence encoding the VP1 capsid protein was amplified with a semi-nested PCR (1D PCR assay), using primers developed specifically for HEVA serotypes [18]. We performed a second PCR assay targeting the genome segment 1A/1B comprising the VP4 and partial VP2 (5′ end) encoding genes if the 1D PCR assay was negative [20]. Visible PCR products after gel electrophoresis were purified and subjected to nucleotide sequencing as described earlier [20]. The sequences determined in the study (n = 117) were submitted to the EMBL/DDBJ/GenBank databases (accession nos. HE572901 to HE573016).

Viral culture

Each specimen underwent at least two passages in human rhabdomyosarcoma (RD) and human lung embryonic fibroblast (MRC5) cell lines. The appearance of viral cytopathic effects was investigated by indirect immunological methods for adenovirus (Ridaquick Rotavirus/Adenovirus Comb®, r-Biopharm, Darmstadt, Germany), herpes simplex types 1 (HSV1) and 2 (HSV2) (Imagen® Herpes simplex virus type 1 and 2; Oxoid, Hants, UK) and cytomegalovirus (monoclonal anti-cytomegalovirus immediate early antigen, Argène, Verniolle, France). The supernatants of cell cultures exhibiting an

EV-like cytopathic effect were collected and stored at −20°C.

Identification of EV serotypes and phylogenetic analyses

Genotyping was carried out by BLAST analysis of 1D sequences with a database of reference sequences and was confirmed by phylogenetic comparison with the EV prototype strains. The nucleotide sequences of strains assigned to serotypes CV-A6, CV-A10, and EV-71 were compared with homologous sequences available in GenBank (up to 27 June 2011), to identify intratypic variants and investigate phylogenetic relationships with strains circulating in distant geographical areas. Multiple sequence alignments were constructed using ClustalW implemented in the BioEdit program. Phylogenetic trees were constructed by the neighbour-joining method. Genetic distances were calculated with the Tamura–Nei model of sequence evolution using MEGA 5 software [21].

Statistical analyses

Data were analysed using SAS statistical software (Version 9.1.3, SAS Institute, Cary, NC, USA). The statistical differences between proportions were tested by chi-squared test or Fisher's exact test. Analysis of variance was used to compare means of age. A *P*-value <0.05 was regarded as statistically significant.

Results

Epidemiological data

From April to December 2010, 222 children (mean age 2.4 years; range 5 weeks–14 years) presenting with HFMD/HA and/or gingivostomatitis had specimens submitted by the sentinel physicians for EV testing. Most of the children (201/222, 90.5%) were seen by private sector paediatricians, who reported an upsurge in these clinical presentations in comparison with earlier years. Infection with EV was diagnosed in 143 (64.4%) children by EV genome detection in throat (n = 95) or buccal (n = 48) swab specimens. An EV strain was isolated in 130 (90.9%) of the 143 EV-positive specimens; RD cells were the only permissive cell line for 73% (95/130). The EV infections mainly occurred between May and July (77%, 111/143) and peaked in June. A second minor wave of EV infections was observed between October and December (Fig. 1).

Demographic and clinical features of patients with EV versus non-EV infections

Testing for EV was negative in 79 patients, and in 25 (31.6%) of these another pathogen was isolated in cell culture. Eighteen children presented with HSV1 infection associated with gingivostomatitis alone (n = 8, 47%) or in association with HFMD (n = 2), HA (n = 3) or both (n = 2). Two patients presented with HA alone. No clinical data was available for one patient. The other identified viruses were cytomegalovirus (CMV, n = 3), rhinovirus (n = 3) and adenovirus (n = 1). Co-infection with EV was diagnosed in three children with HSV1 (n = 1) or CMV (n = 2).

HFMD was only observed in 63/222 patients (28.4%). HA was the predominant clinical presentation (63.8%, 139/222 cases) and was frequently associated with clinical signs of HFMD (67/139, 48%). The mean age of EV-infected patients was lower than that of non-EV-infected patients (p 0.048) (Table 1). The number of males was significantly higher among patients presenting with EV infection (p 0.0009). Most children in both groups had fever. HFMD (p 0.006) or a vesicular rash on hands, feet, knees or buttocks without oral ulcers (p 0.0002) was significantly more frequently observed in EV infections than in non-EV infections.

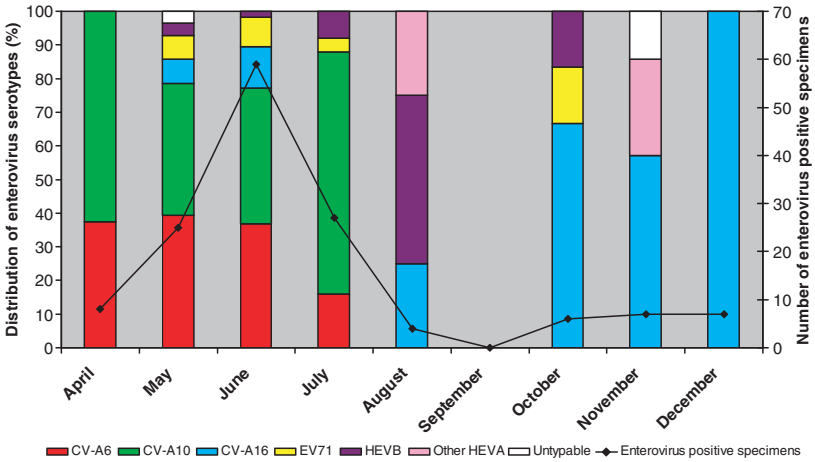

FIG. 1. Distribution of the number of enterovirus-positive specimens and prevalent serotypes by month between April and December 2010. CV-A, coxsackievirus A; EV, enterovirus; HEVB, human enterovirus B species.

TABLE 1. Demographic and clinical features of patients with enterovirus (EV) infections versus non-EV infections

	All patients[a] (*n* = 218)	Enterovirus negative[a] (*n* = 75)	Enterovirus positive (*n* = 143)	p value
Mean age at enrolment, years	2.4	2.8	2.2	0.048
Male/female ratio	1.02	0.55	1.42	0.0009
Fever (temperature >38°C)	167 (76.6)	52 (69.3)	115 (80.4)	0.07
Hand, foot and mouth disease	63 (28.9)	13 (17.3)	50 (35)	0.006
Oral ulcers alone	26 (11.9)	20 (41.7)	6 (9.4)	<0.0001
Vesicular rash on hands, feet, knees or buttocks without oral ulcers	53 (24.3)	7 (9.3)	46 (32.2)	0.0002
Gingivostomatitis	43 (19.7)	21 (28)	22 (15.4)	0.0262
Alone	15 (6.9)	13 (17.3)	2 (1.4)	<0.0001
Herpangina	139 (63.8)	44 (58.7)	95 (66.4)	0.2571
Alone	72 (33)	34 (45.3)	38 (26.6)	0.0051
Digestive signs (abdominal pain, diarrhoea)	30 (13.8)	15 (20)	15 (10.5)	0.053
Ear, nose and throat signs	15 (6.9)	4 (5.3)	11 (7.7)	0.51
Respiratory signs	5 (2.3)	2 (2.7)	3 (2.10)	1

Data are *n* (%) of patients, unless otherwise indicated.
[a]with available clinical data.

HA alone (p 0.0051), oral ulcers and gingivostomatitis alone (p <0.0001) were significantly more frequent in non-EV-infected patients. For the other reported clinical signs, differences were not statistically significant.

Children with HFMD and HA were distributed throughout the city. Environmental data were obtained in 127/222 children (57.2%). Interestingly, 42 (33.1%) children had contact with household members or day-nursery playmates who presented similar symptoms. No statistically significant difference was found between EV-infected and non-EV-infected children, nor between different child-care arrangements (childminder, public preschool, school or home).

Prevalent serotypes and clinical presentations

A definite serotype was identified in 141/143 (98.6%) patients with proven EV infection (Fig. 2). Of the 141 EV strains, 134 (95%) were assigned to a serotype within the HEVA species. The most predominant EVs were CV-A10 (39.9%) and CV-A6 (28%). CV-A16 and EV-71 accounted for 17.5% and 6.3% of cases, respectively, and other HEVA serotypes (CV-A4 and CV-A8) represented 2.1%. HEVB serotypes (echovirus 9, *n* = 2; CV-A9, CV-B4 and CVB-5, and echoviruses 16 and 17, *n* = 1 each) were also identified in 5.6% of cases. Serotype distribution differed between the summer and winter waves of EV infections. Serotypes CV-

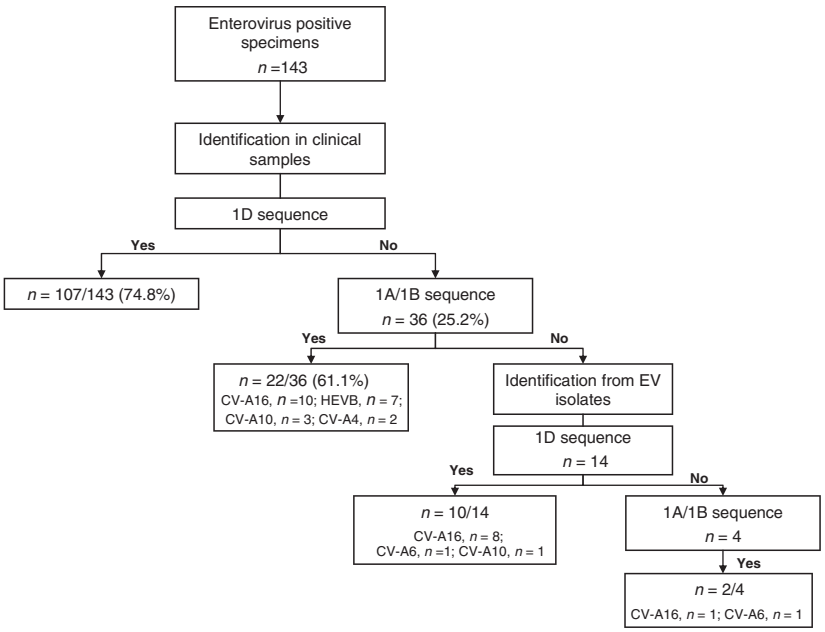

FIG. 2. Methodological approach and results of enterovirus (EV) genotyping. Prospective EV genotyping was attempted in the 143 consecutive patients with EV-positive specimens with the same extract used for the EV genome detection assay. Identification was performed from the complete 1D sequence for 107 (74.8%) specimens and from partial 1A–1B sequence for 22 of the 36 remaining samples. For 12 patients, EV identification was obtained with the strains isolated in cell culture by genotyping of the complete 1D sequence (*n* = 10) or partial 1A–1B sequence (*n* = 2). CV, coxsackievirus; HEVB, human enterovirus B species.

A10 (57/119, 47.9%) and CV-A6 (40/119, 33.6%) predominated from April to July, whereas CV-A16 was involved in 66.7% (16/24) of the EV infections during the second half of the study (Fig. 1). Table 2 compares the demographic and clinical data of patients between the four most prevalent serotypes. HA was more frequent in patients with CV-A6 and CV-A10 infections. Infections with CV-A16 strains were predominantly responsible for strictly defined HFMD and were less frequently associated with fever.

Phylogenetic investigations

Phylogenetic analysis of viral sequences showed that the EV-71 strains belonged to genotype C2 (data not shown). On the basis of analysis of partial 1D sequences, CV-A10 strains were divided into two clusters (18.6% nucleotide differences) (Fig. 3a). Most virus strains belonged to cluster A (mean nucleotide identity of 97.9%) and were closely related to strains isolated during the 2008 HFMD outbreak in Finland. Only one strain (CF203060_FRA10) belonged to cluster B and grouped together with strains isolated in Slovakia (2007) and China (2008–2010). Two genetic groups were detected among the CV-A6 strains (Fig. 3b). Most of the strains were included in cluster A (99.6% nucleotide identity) and displayed close relationships with viruses recovered during the 2008 Finnish outbreak. A number of strains belonged to cluster B (97.2% nucleotide identity), which also included viruses of various geographical origins.

Discussion

The HFMD/HA cases reported here were investigated for the first time in a European country through a prospective observational study involving a sentinel surveillance system composed of practitioners in the private and public sectors. Studies in Europe have focused mainly on EV-infected patients admitted to hospital and so there is scant information on the epidemiology of HFMD/HA cases. For this reason we could not have predicted the large scale of the outbreak before starting the study. It allowed the real-time detection of a dual outbreak caused by CV-A6 and CV-A10 infections, the third in the period 2008–2010 in Europe.

During the 9 months of the survey study, 143/222 (64.4%) children presented with an EV infection. The diagnosis of EV infection was established from throat or buccal swab specimens. Taking vesicular fluid specimens in young children might have been considered too invasive by the children's parents, and was considered difficult to perform by the paediatricians in the private sector. Compared with diagnosis made on the basis of vesicular fluid specimens, we may have underestimated the number of EV infections or identified an EV different from the real aetiological agent of HFMD, because EVs can remain present in the throat for several weeks after an infection. Clinical signs of HFMD and HA were observed in 96/143 and 95/143 children (67.7% and 66.4%), respectively, and were frequently associated. Genotyping of EV was performed directly in clinical specimens for 90.2% of the patients. Using generic primers designed for the amplification of a partial 1D sequence [22], EV identification was effective for 55% of the EV-positive clinical samples during the 2008 Finnish outbreak [10]. The RT-PCR assay used in our study proved to be useful for identifying four other HEVA serotypes in addition to EV-71 [18] and provided an accurate picture of the epidemiology of HEVA. Only a few other RT-PCR assays used for genotyping have been

TABLE 2. Demographic and clinical features associated with the different enterovirus serotypes

	Enterovirus serotypes				
	Coxsackievirus A10 (*n* = 57)	Coxsackievirus A6 (*n* = 40)	Coxsackievirus A16 (*n* = 25)	Enterovirus 71 (*n* = 9)	p value[a]
Mean age at enrolment, years	2.1 ± 1.3	2.0 ± 1.0	2.7 ± 1.3	2.1 ± 1.3	0.1371
Male/female ratio	1.03	3	1.08	3.5	0.0552
Fever (temperature >38°C)	52 (91.2)	33 (82.5)	13 (52)	7 (77.8)	0.0007
Hand, foot and mouth disease	16 (28.1)	12 (30)	18 (72)	3 (33.3)	0.0012
Oral ulcers alone	2 (8.7)	0	0	3 (50)	0.0023
Vesicular rash on hands, feet, knees or buttocks without oral ulcers	14 (24.6)	20 (50)	6 (24)	2 (22.2)	0.0363
Gingivostomatitis	11 (19.3)	4 (10)	6 (24)	1 (11.1)	0.4288
Herpangina	44 (77.2)	28 (70)	12 (48)	3 (33.3)	0.0097
Alone	24 (42.1)	7 (17.5)	1 (4)	0	0.0002
Digestive signs (abdominal pain, diarrhoea)	6 (10.5)	3 (7.5)	1 (4)	2 (22.2)	0.3685
Ear, nose and throat signs	4 (7)	3 (7.5)	1 (4)	0	1
Respiratory signs	0	0	1 (4)	0	0.2595

Data are mean ± standard deviation for age and *n* (%) of patients for clinical symptoms.
[a]Significant variations between the four groups (CV-A10, CV-6, V-A16 and EV71) were evaluated using analysis of variance for age, and chi-squared test or Fisher's exact test for clinical symptoms.

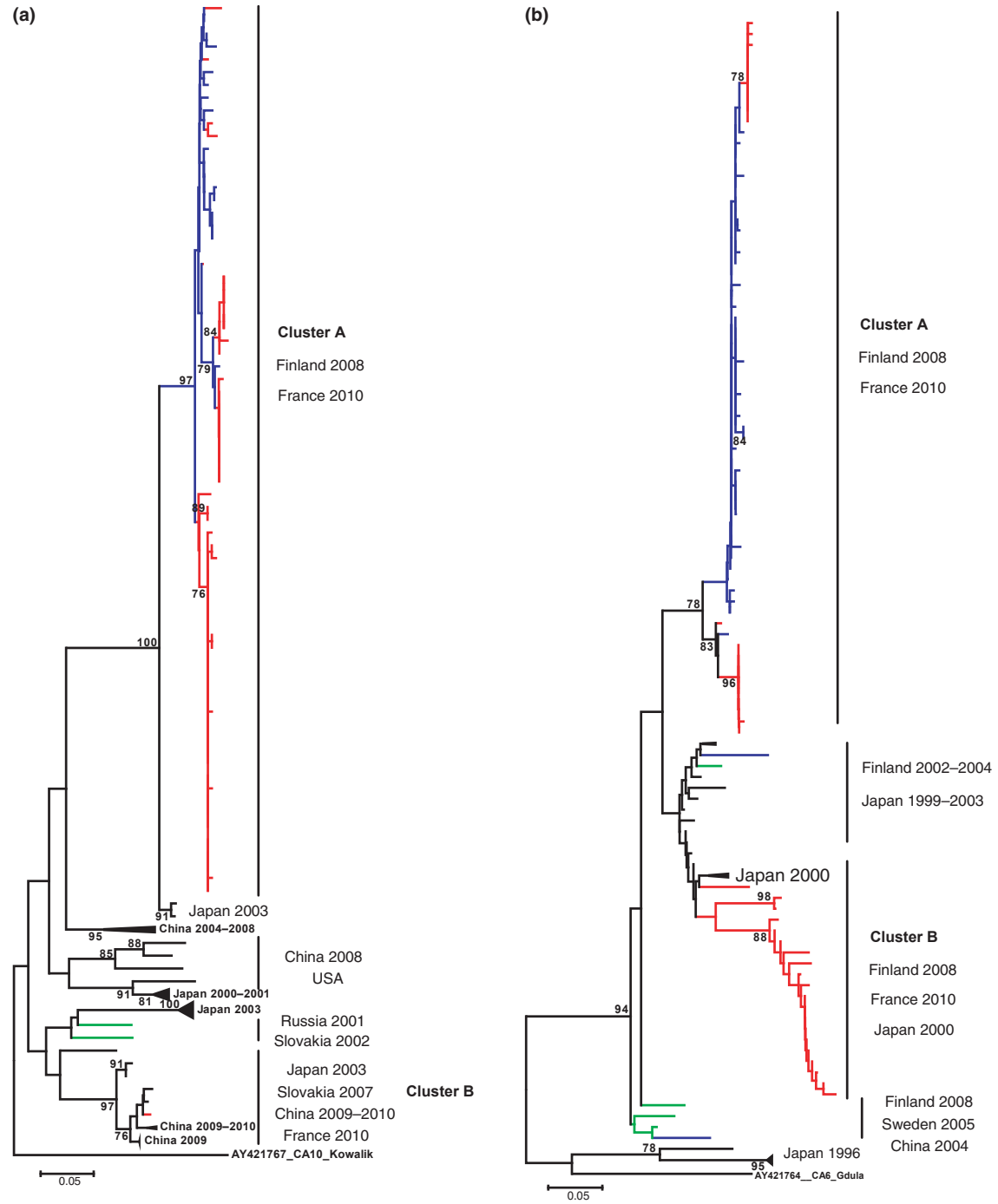

FIG. 3. Phylogenetic trees based on partial VP1 coding sequence of (a) coxsackievirus A10 (246 nucleotides) and (b) coxsackievirus A6 (293 nucleotides). Genetic distances were calculated with the Tamura–Nei model of evolution. The tree was constructed by the neighbour-joining method and validated with 1000 pseudo-replicates. Only bootstrap values of over 70% are shown. Branch length was drawn to the indicated scale (proportion of nucleotide per site). Taxon names have been deleted for clarity (see Supplementary material; Fig. S1). Sequences of strains isolated in Finland are indicated in blue, in France in red, and in other European countries in green.

reported to identify HEVA serotypes from clinical samples [22,23]. In most epidemiological studies of HFMD/HA, EV genotyping relies on virus isolates [3,4] despite the difficulty of growing HEVA serotypes in cell culture [24].

CV-A10 and CV-A6 accounted for two-thirds of all infections in the outbreak. The occurrence of infections with these two serotypes was similar to the epidemiological pattern observed 2 years earlier during a nationwide HFMD

outbreak in Finland [10] and Spain [25]. Of the 1175 EV infections reported to the French Institute for Public Health in 2010, 42 cases were associated with clinical signs of HFMD/HA, of which 59% occurred in south-eastern France. The number of HFMD/HA cases in 2010 was up to ten-fold higher than the yearly number of cases reported during the period 2005–2009. These data indicate a recent increase in the overall circulation of HEVA serotypes. The seasonal patterns of HFMD/HA cases were similar in the national records and during the sentinel study but serotype distribution indicated a higher nationwide prevalence of CV-A16 in the summer months. Comparisons of the clinical presentations associated with CV-A6 and CV-A10 in the three European outbreaks are hampered by differences in study designs as both the Finnish and Spanish studies were retrospective and focused on HFMD cases [10,25]. When surveillance is based on sentinel systems with *a priori* distinct clinical definitions for HFMD and HA, observational studies, from Taiwan [4] and Japan [26], showed that CV-A6 and CV-A10 were associated with HA, whereas CV-A16 and EV-71 were involved in HFMD cases. In addition, as described for earlier outbreaks in Asian countries [4,26], the community outbreak in France occurred in two waves with most cases being diagnosed from April to July. Whatever the EV serotype involved, none of the patients presented with severe or neurological symptoms whereas neurological manifestations (encephalitis and convulsions) were reported in five cases during the Finnish outbreak [10]. At the national level in France, 12 infections with CV-A6 and CV-A10 were reported to the National Institute of Health among children hospitalized during 2010. Only one boy, aged 11 months, presented with convulsions and had a CV-A6 infection. During the same period, 25 EV-71 infections were reported nationally: of the patients with clinical data available (n = 22), eight (36.4%) presented with meningitis. Onychomadesis was a hallmark of earlier HFMD outbreaks in Europe [10,25]. This manifestation was not observed during our study. Although the information was given to the sentinel paediatricians soon after the first identification of CV-A6 and CV-A10 strains, we cannot exclude the possibility that parents did not inform the physicians of the occurrence of nail abnormalities in their children. Considering the close genetic relationships between all CV-A6 and CV-A10 strains recovered in Europe, these clinical differences remain unclear.

The report of three consecutive outbreaks of dual CV-A6 and CV-A10 infections in distinct European countries over the years 2008–2010 is consistent with an epidemiological pattern of emerging virus variants spreading into new geographical areas. CV-A10 was predominant in 2004, 2007 and 2009 in Taiwan [4,9] and in Japan in 2007 [27], and CV-A6 was the most frequent serotype in Japan in 2005 [26] and in Singapore in 2008 [28]. In Europe, a high prevalence of CV-A6 or CVA-10 infections was only reported in Norway [29] and Germany [30] before 2003. In our study, phylogenetic analysis of CV-A10 sequences showed that the predominant lineage in circulation is a genetic variant distinct from that detected over the same period in China or earlier in Europe. Similarly, cluster A CV-A6 viruses constitute a distinct European lineage. This geographical clustering pattern is reminiscent of that seen with EV-71 strains isolated after 2006 in Europe [14,18]. Spreading of genetic variants possibly originating from Asian countries may explain the occurrence of outbreaks in Europe but the factors associated with an apparent dual spreading of both viruses remain unknown. Phylogenetic analysis of the complete 1D sequence will be needed to establish robust genetic relationships between strains from the two geographical areas.

The occurrence of three successive outbreaks over 3 years indicates that HFMD and HA are not rare in Europe. Whether this is a result of an increasing circulation of HEVA serotypes or of improved surveillance needs investigations over longer periods. The overall epidemiology of HFMD/HA caused by HEVA serotypes in European countries resembles that observed in Asian countries, but the lower frequency of EV-71 epidemics in Europe remains unexplained. As there is endemic circulation of EV-71 genogroups C1 and C2 in Europe, prospective diagnosis and collection of clinical data combined with genotyping yield consistent epidemiological data and should be considered for the management of future national outbreaks.

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Transparency Declaration

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Author Contributions

Study concept and design: A. Mirand, C. Henquell, C. Archimbaud, J-L. Bailly, H. Peigue-Lafeuille. Analysis and interpretation of data: A. Mirand. Management of national epidemiological data: D. Antona. Statistical analysis: S. Ughetto. Preparation of the manuscript: A. Mirand, C. Henquell, C. Archimbaud, J-L. Bailly, H. Peigue-Lafeuille.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phylogenetic trees based on partial VP1 coding sequence of coxsackievirus A10 (246 nucleotides; A) and coxsackievirus A6 (293 nucleotides; B) with taxon names.

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